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The Vi Capsular Antigen of *Salmonella enterica* Serotype Typhi Reduces Toll-Like Receptor-Dependent Interleukin-8 Expression in the Intestinal Mucosa

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Human infections with nontyphoidal *Salmonella* serotypes, such as *S. enterica* serotype Typhimurium, are characterized by a massive neutrophil influx in the colon and terminal ileum. In contrast, neutrophils are scarce in intestinal infiltrates of typhoid fever patients. Here, we show that in *S. enterica* serotype Typhi, the causative agent of typhoid fever, expression of the Vi capsular antigen reduced expression of the neutrophil chemoattractant interleukin-8 (IL-8) in host cells. Capsulated bacteria elicited IL-8 expression in polarized human epithelial cells (T84) and human macrophage-like cells (THP-1) in vitro at significantly reduced levels compared to noncapsulated bacteria. Experiments with a human cell line (HEK293) transfected with human Toll-like receptors (TLRs) demonstrated that in the presence of TLR5 or TLR4/MD2/CD14, a noncapsulated serotype Typhi mutant was able to induce the expression of IL-8, while this host response was significantly reduced when cells were infected with the capsulated serotype Typhi wild type. The relevance of these in vitro observations for the interaction of serotype Typhi with its human host was further studied ex vivo using human colonic tissue explants. Expression of IL-8 was detected in human colonic tissue explants infected with serotype Typhimurium or a noncapsulated serotype Typhi mutant. In contrast, infection with the serotype Typhi wild type did not elicit IL-8 expression in colonic tissue explants. Collectively, these data suggest that the scarcity of neutrophils in intestinal infiltrates of typhoid fever patients is due to a capsule-mediated reduction of TLR-dependent IL-8 production in the intestinal mucosa.

Salmonella enterica serotype Typhi is a strictly human-adapted pathogen responsible for some 600,000 deaths and 16,000,000 cases of typhoid fever annually (20). The type of inflammatory infiltrate elicited in the intestines of typhoid fever patients is strikingly different from that observed in patients infected with nontyphoidal *Salmonella* serotypes, such as *S. enterica* serotype Typhimurium. Serotype Typhimurium infections in humans are characterized by a massive neutrophil influx in the terminal ileum and proximal colon (4, 19) and the predominance of neutrophils in stool samples (9). Experiments with cultured human epithelial cells and macrophages suggest that this neutrophil influx is triggered because pathogen-associated molecular patterns (PAMPs) of serotype Typhimurium activate Toll-like receptor (TLR) signaling pathways, thereby resulting in the release of neutrophil chemoattractants (e.g., interleukin-8 [IL-8]) (22, 23, 27, 36). In contrast, serotype Typhi does not elicit a massive neutrophil influx in the human intestine. Instead, mononuclear cells are the predominant cell type identified in intestinal infiltrates (17, 21, 24, 32) and in stool samples of typhoid fever patients (9).

Microarray analysis shows that serotype Typhimurium and purified serotype Typhimurium flagella trigger a classical proinflammatory gene expression program in human colonic carcinoma cells that depends on the recognition of flagella through TLR5 (36). In contrast, serotype Typhi does not trigger this proinflammatory expression profile in human colonic carcinoma cells (36) despite the fact that purified serotype Typhi flagella are potent inducers of proinflammatory cytokine secretion in human monocytes (34, 35). These observations raise the possibility that, unlike serotype Typhimurium, serotype Typhi expresses factors during intestinal invasion that allow it to down-regulate a TLR-mediated host response that leads to neutrophil infiltration. Furthermore, since the mechanism underlying innate immune evasion by serotype Typhi is not operational in serotype Typhimurium, it is likely encoded by a serotype Typhi DNA region that is absent from the serotype Typhimurium genome.

Whole genome sequencing has revealed that serotype Typhi strain CT18 possesses 601 genes present on 82 genetic islands that are absent from the serotype Typhimurium LT-2 chromosome (25). The largest of these genetic islands is a 134-kb DNA region, termed *Salmonella* pathogenicity island 7 (SPI7). A region within SPI7, termed the *viaB* locus, contains genes required for the biosynthesis and the export of the Vi capsular antigen, a linear polymer of α-1,4(2-deoxy)-2-*N*-acetylgalacturonic acid variably *O*-acetylated at the C3 position (10). This capsular polysaccharide is expressed in vitro in macrophages (3) and during human infection, as indicated by protection against typhoid fever following vaccination with the Vi antigen

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TABLE 1. Primers used in this study

Primer use	Sequence (5′ to 3′)
PCR amplification of upstream and downstream regions of *viaB*	TGGGTTAGCCAAAGAC
	GTCGACTGATGTCAAACCTGCAC
	GTCGACGTCACCATCAGTAGCACAACG
	GCACGGACTGTTCCTTGCG
Amplification of *invA*	GTGAAATTATCGCCACGTTCGGGCAA
	TCATCGCACCGTCAAAGGAACC
Amplification of serotype Typhi *fliC*	CAGAACGAAGTGTATCAACCTGTGC
	CCGAAAGAAACTGCTGTAACCG

(16). The Vi antigen has recently been implicated in preventing IL-8 production in human macrophage-like cells (THP-1) (11) and in human colonic carcinoma cells (Caco-2) (31). The Vi antigen is thus a promising candidate for a serotype Typhi-specific virulence factor that blocks the TLR-mediated host responses leading to neutrophil infiltration. Here, we tested this hypothesis using human tissue culture models.

Studies on the pathogenesis of typhoid fever are complicated by the fact that serotype Typhi is a strictly human-adapted pathogen. Serotype Typhi does not cause disease in nonprimate vertebrates or in lower primates (i.e., Rhesus macaques). Although higher primates (i.e., chimpanzees) infected with serotype Typhi develop a typhoid fever-like disease, the scarcity of these animals has effectively prevented the use of this model (7). The lack of a suitable animal model makes it difficult to test whether phenotypes observed during the interaction of serotype Typhi with human cell lines in vitro are representative of changes occurring during the interaction of serotype Typhi with human tissue in vivo. Recently, an ex vivo model using human colonic tissue explants has been adapted for studying serotype Typhimurium interaction with human tissue (8). Here, we used the human colonic tissue explant model to determine the role of the Vi antigen during interaction of serotype Typhi with the human intestinal mucosa ex vivo. These data provided an important link between results from in vitro experiments and clinical observations from typhoid fever patients.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Serotype Typhi strain Ty2 was obtained from the American Type Culture Collection (ATCC 19430). A deletion of the *viaB* region in Ty2 was constructed by allelic exchange. DNA regions flanking the *viaB* locus were PCR amplified (primers are listed in Table 1) and cloned into a suicide plasmid (pGP704). A kanamycin resistance cassette (KSAC) was cloned between the two DNA regions and the resulting plasmid introduced into serotype Typhi strain Ty2 by conjugation. Deletion of the *viaB* region in the resulting strain (STY2) was confirmed by Southern hybridization using a *viaB*-specific DNA probe. A mutation in *invA* was introduced into the STY2 chromosome by inserting a suicide vector (pGP704) carrying an internal fragment of the *invA* gene (Table 1) by homologous recombination to give rise to STY4. A mutation in *fliC* was introduced into the Ty2 chromosome by inserting a suicide vector (pGP704) carrying an internal fragment of *fliC* (Table 1) by homologous recombination to give rise to STY3. Unless indicated otherwise, all serotype Typhi strains were cultured statically in SOB broth (20 g/liter Tryptone, 5 g/liter yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) for optimal expression of the Vi antigen. Strain IR715 is a fully virulent spontaneous nalidixic acid-resistant derivative of serotype Typhimurium strain ATCC 14028. Mutations in *fljB* and *fliC* have been described previously (30) and were introduced into the serotype Typhimurium 14028 chromosome by P22 transduction, yielding strain EHW26. A mutation in *msbB* has been described previously (15) and was introduced into serotype Typhimurium strain 14028 by P22 transduction to yield strain RPW3. All serotype Typhimurium strains were grown statically (for infection of colonic explants, HEK293 cells, or THP-1 cells) or with aeration (for T84 invasion assay) in Luria-Bertani broth.

Flow cytometry. Flow cytometry was performed as described previously (14). DNA was labeled with propidium iodide and Vi antigen detected by labeling cells with rabbit anti-Vi serum (1:250 dilution; BD) and goat anti-rabbit fluorescein isothiocyanate (FITC) conjugate (1:250 dilution; Jackson ImmunoLabs). For each sample, the fluorescence of 10,000 particles (bacterial cells) was measured by flow cytometry (FACSCalibur; Becton Dickinson). A rectangular two-dimensional selection region (bitmap) was drawn such that less than 2% of the control population (serotype Typhimurium) was counted positive for the selected FITC fluorescence intensity.

Human colonic tissue explant model. Colonic biopsies (six biopsies/patient) were collected from patients (between 50 and 80 years of age) undergoing colon cancer screening after we obtained their informed consent and approval from the Institutional Review Boards of Texas A&M University and Scott & White Clinic. Human colonic tissue explants were used as described previously (8) with the following modification. Tissue explants were inserted into Snapwell plates (Corning-Costar) to generate a luminal and a serosal compartment as described previously (6). The luminal compartments of biopsy samples from each individual patient were infected either with approximately 10^7 bacteria (serotype Typhimurium strain IR715, serotype Typhi strain Ty2, or serotype Typhi strain STY2) or with sterile medium. Tissue was snap-frozen 8 h after infection. RNA was extracted from snap-frozen tissue with 1 ml of Tri reagent (Molecular Research Center) according to instructions by the manufacturer. Next, 500 ng of each sample was retrotranscribed in a 50 μl volume (Taqman reverse transcription reagents; Applied Biosystems) and 4 μl of cDNA was used for each real-time (RT)-PCR. RT-PCR with primers (33) to detect human IL-8 (CXCL-8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using SYBR Green (Applied Biosystems) and the GeneAmp 5700 Sequence Detection System. The data were analyzed using the comparative Ct method (Applied Biosystem).

Infection of human cell lines. T84 cells were grown in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Gibco), containing 1.2 g/liter sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate (Gibco), supplemented with 10% fetal calf serum (FCS). To polarize T84 cells, 0.5 ml of medium containing 4×10^5 cells/well was seeded on the apical compartment in 12-mm Transwell plates (12-mm Transwell, polycarbonate membrane, pore size of 0.4 micron; Corning-Costar) and 1.5 ml of medium was added to the basolateral compartment. The medium was changed every other day, and the transepithelial electrical resistance was measured after a week. When the cells reached a transepithelial electrical resistance of at least 1500 Ωcm^2, they were incubated overnight in fresh medium and an assay was performed the following day. For analyzing changes in host gene expression, bacteria (1×10^7/well) were added to the apical side of polarized T84 cells for 1 h and RNA extracted for RT-PCR analysis as described above. For analyzing invasion, bacteria (1×10^7/well) were added to the apical side of polarized T84 cells for 1 h and washed five times with Dulbecco's phosphate-buffered saline 14040 (Gibco) and medium containing 0.1 mg gentamicin/ml was added for 90 min. Intracellular bacteria were quantified by spreading serial 10-fold dilutions of T84 cell lysates (1% Triton X-100) on Luria-Bertani agar plates to determine the number of CFU.

THP-1 cells seeded at approximately 5×10^5 cells/well in 24-well plates containing RPMI 1640 plus 10% FCS were differentiated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h followed by growth without PMA for 4 days. Cells were infected with 1×10^7 bacteria/well for 1 h, washed, and incubated with 0.1 mg/ml gentamicin for 1 h. Subsequently, cells were incubated in medium containing 0.025 mg/ml gentamicin for 4 h. Six hours after infection, RNA extraction and RT-PCR analysis were performed as described above.

HEK293 cells stably transfected with human TLR4/MD2/CD14 or human TLR5 were purchased from Invivogen. Cells were maintained in DMEM with glucose (4.5 g/liter) and 10% FCS supplemented with blasticidin or hygromycin according to the manufacturer's instructions. Cells were seeded (5×10^5 cells/well) in 24-well plates containing DMEM and 10% FCS and infected with 1×10^7 bacteria/well for 4 h. To assess the response of HEK293 cells to purified PAMPs, purified serotype Typhimurium flagella (1 μg/ml) or *S. enterica* serotype Minnesota lipopolysaccharide (LPS, 10 μg/ml; Invivogen) was added and incubated for 4 h. RNA extraction and RT-PCR analysis were performed as described above.

Statistical analysis. For statistical analysis of ratios (i.e., fold increases in IL-8 expression or invasion data expressed as percentage of inoculum), data were

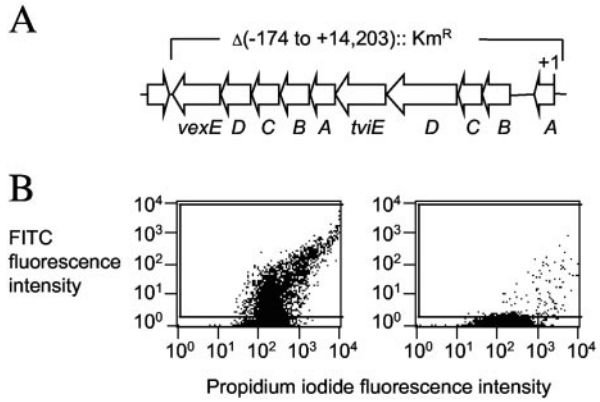

FIG. 1. Construction of a *viaB* deletion mutant of serotype Typhi strain Ty2. (A) Schematic drawing of the DNA region deleted in the serotype Typhi *viaB* mutant. A bracket indicates the size and location of the deletion relative to the first nucleotide of the *tviA* open reading frame. (B) Analysis of the serotype Typhi wild type (left panel) and the serotype Typhi *viaB* mutant (right panel) for expression of the Vi antigen (FITC fluorescence intensity, *y* axes) by flow cytometry. DNA of bacterial cells was labeled with propidium iodide (*x* axes).

transformed logarithmically to calculate geometric means and for statistical analysis. A parametric test (paired Student's *t* test) was used to calculate whether differences in fold increases of IL-8 expression or invasion between treatment groups were statistically significant.

RESULTS

Deletion of the *viaB* region of serotype Typhi. To determine whether the Vi antigen plays a role in preventing the production of neutrophil chemoattractants during infection, we constructed a derivative of serotype Typhi strain Ty2 carrying a precise deletion of the *viaB* region by allelic exchange (STY2) (Fig. 1A). Unlike the serotype Typhi wild type (Ty2), the serotype Typhi *viaB* mutant (STY2) could not be agglutinated with anti-Vi antiserum (data not shown). Expression of the Vi antigen in strains Ty2 and STY2 was further investigated by flow cytometry. Cells were labeled with rabbit anti-Vi antiserum and goat anti-rabbit IgG FITC conjugate for detection of the Vi antigen (*y* axes) and propidium iodide for detection of DNA (*x* axes). The gate for detection of Vi expression was set such that serotype Typhi cells were considered positive for expressing the Vi antigen when their FITC fluorescence intensity exceeded that of all but a small fraction (less than 2%) of a Vi antigen negative control population (i.e., serotype Typhimurium cells). Using this gate, expression of the Vi antigen was detected by flow cytometry in the serotype Typhi wild type (Ty2) but not the serotype Typhi *viaB* mutant (STY3) (Fig. 1B).

The Vi antigen reduces IL-8 expression in human intestinal epithelial cells. Interaction with the intestinal mucosa during serotype Typhi and serotype Typhimurium infection is initiated by invasion of the intestinal epithelium. Early mechanisms by which the Vi antigen may diminish IL-8 production in the intestinal mucosa were thus investigated using the polarized epithelial cells derived from a human colonic carcinoma (T84 cells) as a model.

Serotype Typhimurium is a biphasic serotype in which a phase variation mechanism results in the expression of flagella that are composed of either flagellin protein FliC (H1 phase) or FljB (H2 phase). Infection of polarized T84 cells with the serotype Typhimurium wild type (IR715) resulted in an approximately sevenfold increase in IL-8 expression ($P < 0.05$) (Fig. 2A). Unlike the serotype Typhimurium wild type, a nonflagellated serotype Typhimurium mutant (*fliC fljB* mutant) did not elicit IL-8 expression in polarized T84 cells (Fig. 2A). These findings were consistent with the previous observation that IL-8 production in T84 cells is triggered by the recognition of serotype Typhimurium flagella through TLR5 (36). A noninvasive serotype Typhimurium strain (*invA* mutant) caused levels of IL-8 expression similar to those of the isogenic wild type (Fig. 2A), which was consistent with earlier reports that bacterial invasion does not contribute to chemokine production in polarized T84 cells (36).

Serotype Typhi is a monophasic serotype which encodes only a single flagellin gene, *fliC*. Infection of polarized T84 cells with the serotype Typhi wild type (Ty2) or a serotype Typhi *fliC* mutant (STY3) resulted in only a modest (less that twofold) increase in IL-8 expression (Fig. 2B). The serotype Typhi *viaB* mutant (STY2) elicited significantly ($P < 0.05$) higher IL-8 expression in T84 cells than the serotype Typhi wild type (Ty2) or the *fliC* mutant (STY3) (Fig. 2B). Expression of the FliC flagellin protein of serotype Typhi could be detected in the wild type (Ty2) and the *viaB* mutant (STY2) by both slide agglutination and Western blotting using anti-FliC antiserum, suggesting that the expression of a capsule did not prevent the expression of flagella (data not shown). Expression of the Vi capsular antigen reduced the invasiveness of serotype Typhi for polarized T84 cells, as shown by a gentamicin protection assay (Fig. 2C). However, the ability to invade T84 cells did not affect the IL-8 expression elicited by serotype Typhi because a noninvasive, noncapsulated strain (*viaB invA* mutant strain STY4) and an invasive noncapsulated strain (*viaB* mutant strain STY2) both elicited similar levels of IL-8 expression (Fig. 2B and C). Collectively, these data provided evidence that induction in T84 cells of IL-8 expression could be significantly reduced by expression of the Vi antigen.

The Vi antigen reduces IL-8 expression in human macrophage-like cells. Subsequent to penetration of the intestinal epithelium, serotype Typhimurium is found exclusively intracellularly in the lamina propria within mononuclear cells and neutrophils (28). This interaction with phagocytes in the lamina propria likely contributes to chemokine production in tissue, since human macrophages produce IL-8 in response to stimulation with serotype Typhimurium PAMPs, including LPS (TLR4/MD-2/CD14 ligand) and flagella (TLR5 ligand) in vitro (22, 23, 27). Late mechanisms by which the Vi antigen may reduce IL-8 production in the human lamina propria were thus investigated using human macrophage-like (THP-1) cells as a model.

Infection with the serotype Typhimurium wild type (IR715) induced the expression of IL-8 in THP-1 cells (Fig. 2D). This response was significantly ($P < 0.05$) reduced in THP-1 cells infected with the serotype Typhi wild type (Ty2) or with a serotype Typhimurium mutant expressing a form of LPS in which acylation of lipid A is reduced, thereby preventing recognition through TLR4/MD2/CD14 (*msbB* mutant). In contrast, the serotype Typhi *viaB* mutant (STY2) elicited IL-8 production at levels similar to those of the serotype Typhi-

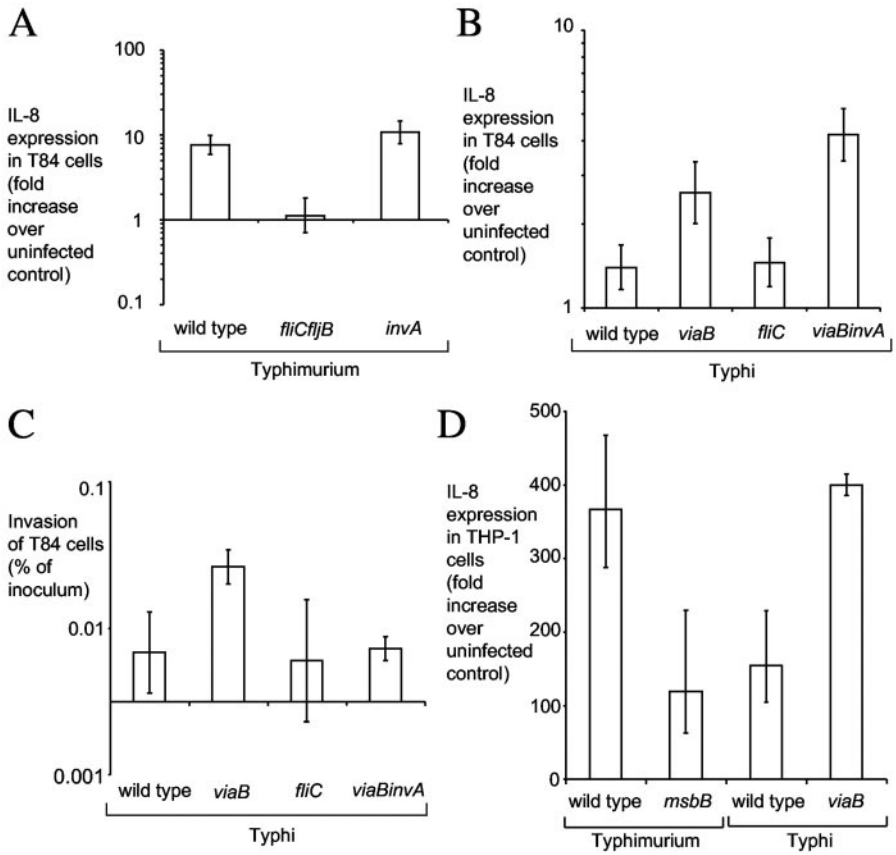

FIG. 2. Interaction of capsulated and noncapsulated bacteria with polarized human colonic epithelial (T84) cells and human macrophage-like (THP-1) cells. (A) IL-8 expression induced by serotype Typhimurium in polarized T84 cells. (B) IL-8 expression induced by serotype Typhi in polarized T84 cells. Bacteria (1×10^7/well) in panels A and B were added to the apical side of polarized T84 cells, and RNA was extracted 1 h after infection. (C) Serotype Typhi invasion of polarized T84 cells. Bacteria (1×10^7/well) were added to the apical side of polarized T84 cells for 1 h, washed, and incubated in medium containing 0.1 mg gentamicin/ml for an additional 90 min before we determined the number of intracellular bacteria. (D) IL-8 expression induced by serotype Typhi and serotype Typhimurium in THP-1 cells. Cells were infected with 1×10^7 bacteria/well for 1 h, washed, and incubated with gentamicin for an additional 5 h prior to RNA extraction. All data are shown as geometric means from three independent experiments ± standard deviations.

murium wild type (IR715). Similar numbers of the serotype Typhi wild type (Ty2) and the *viaB* mutant (STY2) were recovered from THP-1 cells at the time RNA was collected, suggesting that differences in IL-8 production were not due to differences in bacterial load (data not shown). Furthermore, a significant ($P < 0.05$) increase in IL-8 production elicited by the serotype Typhi *viaB* mutant (STY2) compared to the wild type (Ty2) was also observed when above experiment was repeated with formaldehyde-killed bacteria (data not shown). These data provided evidence that expression of the Vi antigen attenuates IL-8 production in THP-1 cells.

The Vi antigen reduces IL-8 expression induced by TLR signaling. To directly test whether the expression of capsular polysaccharide reduces TLR signaling by host cells, we measured IL-8 expression by human embryonic kidney (HEK293) cells transfected with human TLR5 (HEK293-TLR5 cells) or human TLR4/MD2/CD14 (HEK293-TLR4 cells) in response to bacterial infection. Induction of IL-8 expression in untransfected HEK293 cells infected with serotype Typhi (data no shown) or commercially obtained purified serotype Typhimurium flagella (Fig. 3A) was negligible. IL-8 expression could be induced in HEK293-TLR4 cells by adding purified LPS. Purified serotype Typhimurium flagella also induced IL-8 expression in HEK293-TLR4 cells, presumably because the preparation contained LPS contamination. Addition of purified serotype Typhimurium flagella to HEK293-TLR5 cells induced IL-8 expression, but addition of purified LPS did not (Fig. 3A). These data confirmed that IL-8 production in HEK293-TLR5 cells is due to the recognition of flagella by TLR5 and in HEK293-TLR4 cells to the recognition of LPS by the TLR4/MD2/CD14 receptor complex.

Flagella did not contribute to the innate immune recognition of serotype Typhi in HEK293-TLR4 cells because the wild type and the *fliC* mutant elicited similar levels of IL-8 expression. The noncapsulated serotype Typhi *viaB* mutant elicited significantly ($P < 0.05$) higher levels of IL-8 expression in HEK293-TLR4 cells than the capsulated serotype Typhi wild type (Fig. 3B), suggesting that the Vi antigen attenuates host responses elicited through TLR4.

The serotype Typhi wild type (Ty2), but not the serotype Typhi *fliC* mutant (STY3), elicited IL-8 expression in HEK293-TLR5 cells (Fig. 3C), thus confirming the notion that IL-8

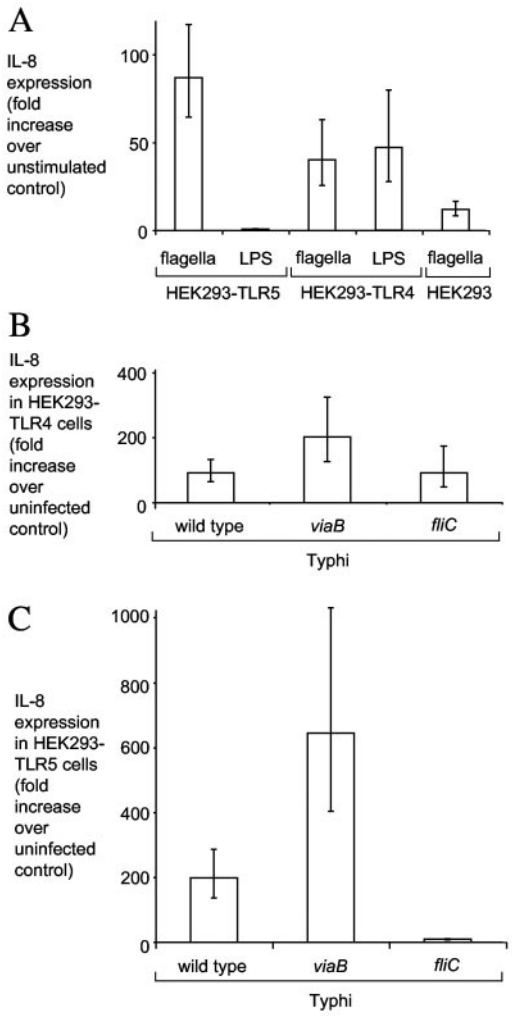

FIG. 3. IL-8 expression elicited by serotype Typhi or purified PAMPs in human embryonic kidney (HEK293) cells stably transfected with human TLRs. (A) IL-8 expression induced by purified PAMPs in HEK293 cells and HEK293 cells transfected with TLRs. Cells were incubated for 4 h with purified serotype Typhimurium flagella (1 μg/ml) or serotype Minnesota LPS (10 μg/ml) prior to RNA extraction. (B) IL-8 expression induced by serotype Typhi in HEK293 cells transfected with human TLR4/MD2/CD14. (C) IL-8 expression induced by serotype Typhi in HEK293 cells transfected with human TLR5. Cells in panels B and C were infected with 1×10^7 bacteria/well for 4 h prior to RNA extraction. All data are shown as geometric means from three independent experiments ± standard deviation.

production in this cell line is due to the stimulation of TLR5 by serotype Typhi flagella. The noncapsulated serotype Typhi *viaB* mutant (STY2) elicited significantly ($P < 0.05$) higher levels of IL-8 expression in HEK293-TLR5 cells than the capsulated serotype Typhi wild type (Ty2) (Fig. 3C). These results provided evidence that IL-8 expression elicited by the recognition of flagellin through TLR5 is attenuated by expression of the Vi antigen in serotype Typhi.

Collectively, these data demonstrated that expression of the Vi antigen diminishes a response (i.e., IL-8 expression) that is induced in host cells when serotype Typhi PAMPs (i.e., flagella and LPS) are recognized by their cognate pathogen recognition receptors (i.e., TLR5 and TLR4).

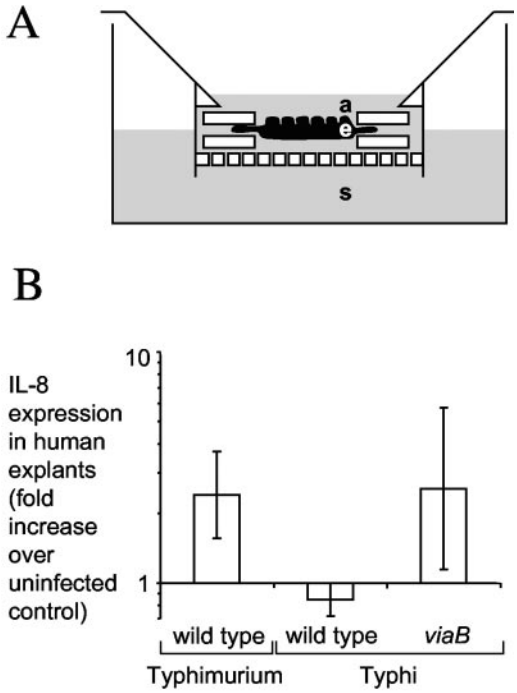

FIG. 4. IL-8 expression elicited by serotypes Typhi and Typhimurium in human colonic tissue explants. (A) Human colonic tissue explant model. Tissue explants (e) were inserted into Snapwell plates to generate luminal (a) and serosal (s) compartments. RNA was collected 8 h after infection of the luminal compartment. (B) Fold increase of IL-8 expression in human colonic tissue explants upon infection with capsulated or noncapsulated bacteria. Data are shown as geometric means from three independent experiments ± standard deviations.

The Vi antigen prevents IL-8 expression in human colonic tissue explants. Serotype Typhi is a strictly human-adapted pathogen, causing disease only in humans and higher primates (i.e., chimpanzees) (7). To investigate the role of the Vi antigen in a relevant model that closely resembles host pathogen interaction in vivo, we studied the infection of human colonic tissue explants. Human colonic tissue explants were obtained from patients undergoing colon cancer screening, sandwiched between circular Plexiglas pieces containing a central hole, and inserted into Costar Snapwell plates (Fig. 4A). Using samples from one individual patient, the top (luminal) compartment of each Snapwell plate was infected with either serotype Typhimurium, serotype Typhi, the serotype Typhi *viaB* mutant, or a mock control (sterile medium). The experiment was repeated with biopsy samples from three individual patients. Eight hours after infection, RNA was extracted from tissue explants and IL-8 expression quantified by RT-PCR.

While serotype Typhimurium elicited the expression of IL-8 in human colonic explants, serotype Typhi did not (Fig. 4B). Deletion of the *viaB* region resulted in a significant increase in IL-8 expression elicited by serotype Typhi (Fig. 4B). There was variation in the level of IL-8 expression between samples from different patients. However, IL-8 expression in samples from each individual patient was increased after infection with the serotype Typhimurium wild type and the serotype Typhi viaB mutant, while in each case no increase (compared to the mock

infected control) in IL-8 expression was detected in tissue infected with the serotype Typhi wild type. These data suggested that mechanisms by which the Vi antigen reduces the production of neutrophil chemoattractants in human tissue culture cells in vitro abrogate IL-8 expression during infection of the human intestinal mucosa.

DISCUSSION

Intestinal biopsy samples collected from humans infected with serotype Typhimurium contain an inflammatory infiltrate that is dominated by neutrophils (4, 19). In contrast, neutrophils are sparse or absent in intestinal biopsies from typhoid fever patients (17, 32). Little progress has been made in understanding why serotype Typhimurium and serotype Typhi cause different types of inflammatory infiltrates in the human intestine, in part because higher primates (i.e., chimpanzees) are the only animal model for studying infection with the strictly human-adapted serotype Typhi. Dougan and coworkers recently established a human colonic tissue explant model for studying the interaction of serotype Typhimurium with the intestinal mucosa (8). Here, we show that colonic tissue explants can be used to model the differences in the inflammatory responses elicited by serotype Typhimurium and serotype Typhi in the human intestine. That is, infection with serotype Typhimurium induced expression of the neutrophil chemoattractant IL-8 in human colonic explants, which correlated well with the neutrophilic infiltrate observed in the intestinal biopsy samples collected from patients infected with serotype Typhimurium (4, 19). Similarly, the absence of IL-8 expression in human colonic explants infected with serotype Typhi correlated well with the reported scarcity of neutrophils in intestinal biopsy samples from typhoid fever patients (17, 32). In the absence of a convenient animal model for studying the interaction of serotype Typhi with the intestinal mucosa, human colonic tissue explants are a powerful and necessary tool for verifying that data obtained using simplified models (i.e., in vitro experiments with human cell lines) can be reproduced in the complex environment of human tissue.

In vitro studies suggest that serotype Typhimurium causes a neutrophil influx in the human intestinal mucosa because its PAMPs (i.e., flagella and LPS) activate TLR signaling pathways in host cells (i.e., in epithelial cells and macrophages), which results in the release of neutrophil chemoattractants (e.g., IL-8 and GROα). Tissue culture models further suggest that, during serotype Typhimurium infection, IL-8 expression in the human intestinal mucosa is induced by a TLR5-mediated response in the intestinal epithelium (36) and by a TLR4- and TLR5-mediated response in macrophages of the lamina propria (22, 23, 27). Events leading to diarrhea and neutrophil infiltration in the intestines of patients infected with serotype Typhimurium can be modeled in vivo using serotype Typhimurium infection of calves (29, 38). Analysis of GROα expression elicited by serotype Typhimurium in bovine ligated ileal loops in situ shows that at 1 h after infection, epithelial cells are the predominant cell type expressing this neutrophil chemoattractant (37). At 1 h after infection of bovine ligated ileal loops, the bulk of the bacteria is located either in the intestinal lumen or within epithelial cells (26, 28). By 4 h after infection, serotype Typhimurium clears the bovine ileal epithelium and is detected by electron microscopy within neutrophils and mononuclear cells in the lamina propria (28). The in vitro and in vivo observations described above imply that at later time points after serotype Typhimurium infection (≥4 h), phagocytes in the lamina propria may be a source of CXC chemokine production. Our data show that the fold increase in IL-8 expression induced during the serotype Typhimurium infection of macrophage-like cells in vitro (>300-fold) is far greater than that elicited in human epithelial cells (approximately sevenfold). However, further in situ analysis is required to determine the relative contribution of epithelial cells and mononuclear cells to chemokine production in the intestinal mucosa at later times after infection with serotype Typhimurium.

Tissue culture experiments show that the release of IL-8 in cultured epithelial cells or macrophages infected with serotype Typhi is markedly decreased compared to that seen in response to serotype Typhimurium infection (11, 18, 36). However, it is not obvious why serotype Typhi does not trigger the release of IL-8 in the human intestinal mucosa, because serotype Typhi is an invasive pathogen and purified serotype Typhi flagella or purified serotype Typhi LPS are potent inducers of IL-8 secretion in human monocytes (1, 22, 35).

Here, we show that, compared to serotype Typhimurium, serotype Typhi elicits reduced IL-8 expression in cultured epithelial cells (T84) and macrophages (THP-1) because it expresses the Vi antigen. These data confirmed a recent report in which capsulated and noncapsulated clinical isolates of serotype Typhi were compared in order to investigate the role of the Vi antigen in modulating IL-8 expression in a human epithelial cell line (Caco-2) (31). Whole genome sequencing of serotype Typhi strains Ty2 and CT18 illustrates that different serotype Typhi clinical isolates exhibit marked genetic differences. For example, serotype Typhi Ty2 possesses 29 genes that are absent from the serotype Typhi CT18 genome and CT18 possessing 84 genes that are absent from the Ty2 genome (5). Furthermore, 9 genes that are intact in Ty2 are pseudogenes in the CT18 genome while 11 genes that are intact in CT18 are pseudogenes in the Ty2 genome (5). The presence of numerous genetic differences makes it difficult to draw compelling conclusions from a comparison between capsulated and noncapsulated serotype Typhi clinical isolates. To directly test whether expression of a capsular polysaccharide prevents the production of neutrophil chemoattractants, we compared the IL-8 expression elicited in human cell lines during infection with a serotype Typhi wild-type isolate (Ty2) with that elicited by its isogenic *viaB* deletion mutant (STY2). Our data show that the Vi antigen is a serotype Typhi virulence factor that reduces TLR5 and TLR4/MD2/CD14-mediated IL-8 expression in host cells. These data implied that, in the absence of the Vi antigen, serotype Typhi PAMPs (i.e., flagella and LPS) efficiently induce TLR-dependent IL-8 expression in host cells. Unlike the serotype Typhi wild type (Ty2), a serotype Typhi *viaB* mutant (STY2) was able to elicit IL-8 expression in human tissue culture cells at levels similar to those seen during serotype Typhimurium infection. Similarly, the serotype Typhimurium wild type and the serotype Typhi *viaB* mutant elicited IL-8 expression in human colonic tissue explants, while no IL-8 expression was elicited during infection with the capsulated serotype Typhi wild type. These data demonstrate for the first time that Vi antigen-mediated suppression of IL-8 production

can be observed in a model (i.e., human tissue explants) that closely resembles host pathogen interaction in vivo, thereby providing support for the idea that the Vi antigen is responsible for the differences in inflammatory infiltrates elicited by serotype Typhi and serotype Typhimurium in the human intestinal mucosa.

While this study was in progress, Sharma and Qadri showed that purified Vi antigen binds to prohibitin at the surface of intestinal epithelial cells (Caco-2 cells) (31). Furthermore, purified Vi antigen reduces extracellular signal-regulated kinase phosphorylation and IL-8 production in response to the PMA stimulation (TLR stimulation was not investigated in this study) of Caco-2 cells (31). The Vi antigen-mediated inhibition of the mitogen-activated protein kinase pathway described by Sharma and Qadri is likely a mechanism contributing to the Vi antigen-mediated inhibition of TLR signaling and IL-8 production described in this study. A capsule-mediated inhibition of TLR signaling provides an attractive explanation for the scarcity of neutrophils in the intestinal infiltrates of typhoid fever patients.

Our data suggest that a noncapsulated serotype Typhi strain would show a greater propensity to elicit neutrophil infiltration in the intestinal mucosa because its PAMPs (i.e., LPS and flagella) would activate TLR signaling pathways in host cells. However, a neutrophilic infiltrate is not noted during infection of volunteers with a serotype Typhi *galE viaB* vaccine candidate (12, 13). A likely explanation for this finding is that a strain carrying a mutation in *galE*, a gene encoding the enzyme uridine-5′-diphospate galactose epimerase required for the biosynthesis of the LPS outer core, is not able to elicit neutrophil infiltration. The finding that a serotype Typhimurium *galE* mutant is unable to elicit neutrophil influx and fluid accumulation in bovine ligated ileal loops, an animal model for human gastroenteritis, provides experimental support for this idea (2).

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